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The extremo-α-carbonic anhydrase (CA) from *Sulfurihydrogenibium azorense*, the fastest CA known, is highly activated by amino acids and amines

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ABSTRACT

The α-carbonic anhydrase (CA, EC 4.2.1.1) from the extremophilic bacterium *Sulfurihydrogenibium azorense* (SazCA) was recently shown to be the fastest CA known. Here we investigated this enzyme for its activation with a series of amino acids and amines. The best SazCA activators were D-Phe, L-DOPA, L- and D-Trp, dopamine and serotonin, which showed activation constants in the range of 3–23 nM. L- and D-His, L-Phe, L-Tyr, 2-pyridyl-methylamine and L-adrenaline were also effective activators (K_As in the range of 62–90 nM), whereas D-Dopa, D-Tyr and several heterocyclic amines showed activity in the micromolar range. The good thermal stability, robustness, very high catalytic activity and propensity to be activated by simple amino acids and amines, make SazCA a very interesting candidate for biomimetic CO_2 capture processes.

Carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous metalloenzymes present in all life kingdoms.[1] They are divided into five evolutionary distinct classes, the α-, β- and γ-, as well as the recently discovered δ- and ζ- classes.[1–5] In Bacteria, CAs belonging to the first three classes have been identified so far, many of them being thoroughly investigated recently, in the search of novel biomedical or biotechnological applications.[6–12] Indeed, many pathogenic bacteria, such as *Brucella* spp., *Mycobacterium tuberculosis, Streptococcus* spp., *Helicobacter pylori*, *Salmonella enterica, Vibrio cholera,* etc, encode such enzymes [6–12] which have been cloned, purified and investigated for their inhibition profiles, in the search for antibiotics with a novel mechanism of action. [6–12] In fact, it has been demonstrated that in many such bacteria, these enzymes are essential for the life cycle of the organism.[6] Recently, this group identified and characterized two α-class CAs from two extremophilic bacteria belonging to the genus *Sulfurihydrogenibium*, that is, SspCA from *Sulfurihydrogenibium yellowstonense* YO3AOP1,[13] and SazCA, from *S. azorense*.[14] These enzymes were called 'extremo-α-CAs' due to their peculiar features: they are highly thermostable, maintaining a good catalytic activity even after heating for a prolonged period (more than 3 h) to 100 °C,[13,14] and they are also highly catalytically effective catalysts for the CO_2 hydration reaction. In fact, SazCA is the fastest CA known to date, and the second most efficient enzyme (after superoxide dismutase), with a k_{cat}/K_M value of 3.5×10^8 M^{-1} s^{-1}.[14] SspCA also showed a good catalytic activity for the same reaction, with a k_{cat} value of 9.35×10^5 s^{-1} a k_{cat}/K_M value of 1.1×10^8 M^{-1} s^{-1}, showing that the 'extremo-α-CAs' are indeed among the most effective CAs known to date.[12,13]

We have recently reported that a multitude of physiologically important compounds, such as biogenic amines (histamine, serotonin, and catecholamines), amino acids, oligopeptides, or small proteins among others, work as efficient activators of many α-CAs.[15] By means of electronic spectroscopy, X-ray crystallography and kinetic measurements, it has been proved that CAAs bind within the enzyme active cavity at a site distinct of the inhibitor or substrate binding-ones, participating thereafter in the rate-limiting step of the catalytic cycle, the proton transfer reaction between the active site and the environment.[15–18] We also reported very recently the first activation study of a bacterial α-CA, that is, SspCA, with a range

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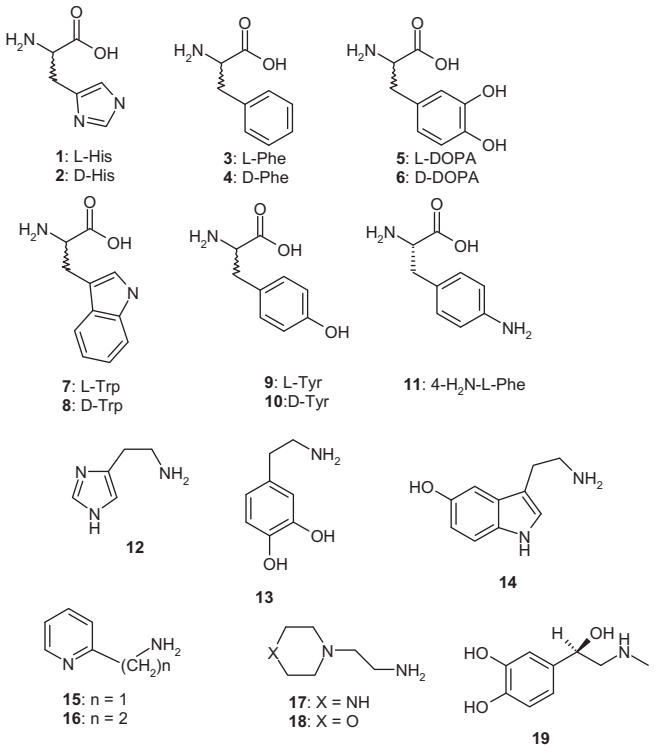
of amino acids and amines,[19] observing that this bacterial highly catalytically efficient enzyme is also very prone to be activatable by this class of modulators of activity. Here we report an activation study of the fastest CA known to date, SazCA, which, as mentioned above, is another extremo-CA, phylogenetically related to SspCA.[14]

L-/D-Amino acids and amines of types **1–19** investigated as SazCA activators were commercially available and were investigated earlier as activators of all human CA isoforms (from hCA I to hCA XV),[15–18] and more recently as SspCA activators.[19]



Kinetic experiments for the CA catalyzed physiological reaction (i.e., carbon dioxide hydration to bicarbonate and a proton) (Table 1) showed that as for the widely investigated human isoforms hCA I and II,[15–18] the activators of the amino acid or amine type, enhance k_{cat} of the enzyme, with no effect on K_M. Indeed, as observed from data of Table 1, L- or D-Phe (compounds **3** and **4**) at a concentration of 10 μM, produced a notable enhancement of k_{cat} for SazCA. In fact this kinetic parameter was 3.4 times higher in the presence of L-Phe and 7.5 times higher (compared to the reaction without activator) in the presence of the more effective activator D-Phe, with values in the range of 1.5–3.3 × 10^7 s^{-1} (Table 1). It may be observed that these are the highest enhancement of k_{cat} ever observed for the activation of a CA (up to now, hCA I and II were the most activatable CAs,[15–18] and only recently a very potent such effect has been reported for SspCA).[19]

SazCA activation constants (K_As) for a series of structurally related amino acids and amines of types **1–19** are shown in Table 2.[20,21] The activation constants for the ubiquitous isozymes hCA I and hCA II are also provided for comparison. Similarly with the inhibition constant K_I, (for the enzyme inhibitors),[4,5] the activation constant K_A measures the affinity of the activator for the enzyme. The lower this parameter is, stronger is the activator against the corresponding isoform.[15–19] Compounds **1–19** were shown earlier to act as activators of all the mammalian isozymes hCA I to mCA XV (h = human, m = murine isoform).[15–18] All these derivatives incorporate protonatable moieties of the primary amine or heterocyclic amine type (or both of them) in their molecules, being thus able to participate in proton transfer processes leading to the generation of the nucleophilic species of the enzyme, with the hydroxide anion coordinated to the active site zinc ion. It should be noted that the amines included in our study possess aminoethyl or aminomethyl moieties, in addition to aromatic/heterocyclic groups, the last of which usually incorporate nitrogen atoms that can be protonated at pH values in the physiological range, as investigated earlier in some detail.[21,22]

Data of Table 2 show the following structure–activity relationship for the activation of SazCA with amines and amino acids of type **1–19**:

(i) Very effective activation of SazCA has been evidenced with a large number of amino acids (suchas D-Phe **4**, L-DOPA **5**, L-Trp **7**, D-Trp **8**) and two amines, dopamine **13** and serotonin **14**, which showed activation constants in the low nanomolar

Table 1
Kinetic paramaters for the activation of hCA isozymes I, II, as well as the bacterial one SspCA and SazCA with L- and D-Phe, at 25 °C and pH 7.5, for the CO_2 hydration reaction.[20] data for hCA I and II are from Ref. [15] whereas for SspCA from Ref. [19]

Isozyme	k_{cat}[a] (s^{-1})	$(k_{cat})_{L\text{-Phe}}$[b] (s^{-1})	$(k_{cat})_{D\text{-Phe}}$[b] (s^{-1})	K_A[c] (μM)	
				L-Phe	D-Phe
hCA I[d]	2.0×10^5	19.8×10^5	2.3×10^5	0.07	86
hCA II[d]	1.4×10^6	5.7×10^6	5.2×10^6	0.013	0.035
SspCA[e]	9.35×10^5	1.4×10^7	4.1×10^6	0.008	5.13
SazCA[e]	4.40×10^6	1.5×10^7	3.3×10^7	0.062	0.009

[a] Observed catalytic rate without activator. K_M values in the presence and the absence of activators were the same for the various CA isozymes (data not shown).
[b] Observed catalytic rate in the presence of 10 μM activator.
[c] The activation constant (K_A) for each isozyme was obtained as described earlier,[20,21] and represents the mean from at least three determinations by a stopped-flow, CO_2 hydrase assay method.[20] Standard errors were in the range of 5–10% of the reported values.
[d] Human recombinant isozymes.
[e] Bacterial recombinant isozymes.

Table 2
Activation constants of hCA I/hCA II (cytosolic isozymes), and the bacterial extremo-CAs SspCA and SazCA with amino acids and amines **1–19**. Data for hCA I and II activation with these compounds (except **9** and **10**) are from Ref.[15]

No.	Compound	K_A[a] (μM)			
		hCA I[b]	hCA II[b]	SspCA[c]	SazCA[c]
1	L-His	0.03	10.9	0.11	0.071
2	D-His	0.09	43	0.012	0.090
3	L-Phe	0.07	0.013	0.008	0.062
4	D-Phe	86	0.035	5.13	0.009
5	L-DOPA	3.1	11.4	0.09	0.004
6	D-DOPA	4.9	7.8	0.43	0.89
7	L-Trp	44	27	0.007	0.023
8	D-Trp	41	12	0.002	0.003
9	L-Tyr	0.02	0.011	0.001	0.052
10	D-Tyr	0.34	0.087	0.83	0.11
11	4-H2N-L-Phe	0.24	0.15	0.97	0.09
12	Histamine	2.1	125	0.080	0.10
13	Dopamine	13.5	9.2	0.037	0.011
14	Serotonin	45	50	0.021	0.007
15	2-Pyridyl-methylamine	26	34	0.10	0.081
16	2-(2-Aminoethyl)pyridine	13	15	0.33	0.34
17	1-(2-Aminoethyl)-piperazine	7.4	2.3	0.09	0.076
18	4-(2-Aminoethyl)-morpholine	0.14	0.19	0.10	1.15
19	L-Adrenaline	0.09	96	0.68	0.074

[a] Mean from three determinations by a stopped-flow, CO_2 hydrase method.[20] Standard errors were in the range of 5–10% of the reported values.
[b] Human recombinant isozymes, stopped flow CO_2 hydrase assay method.[20]
[c] Full length, bacterial recombinant enzyme, stopped flow CO_2 hydrase assay method.[20]

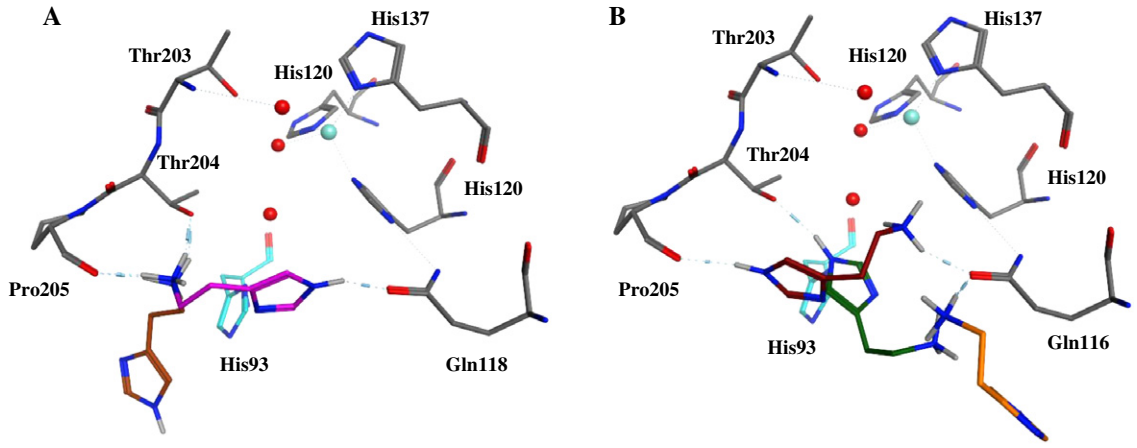


Figure 1. Docked poses of histamine (N^{τ}–H tautomer) in the SazCA homology model based on the crystal structure of *Neisseria gonorrhoeae* CA. Histamine interacts with Gln116, Thr204 and/or Pro205 at the entrance of the active site and is therefore water exposed. The histamine binding poses are shown in different colours for clarity. All poses that form hydrogen bonds with Thr204/Pro205 through the histamine ethylamine tail are depicted in Panel A and all poses that form hydrogen bonds between the ethylamine tail and Gln116 are depicted in Panel B. His93 (His64 in hCA II) is depicted in turquoise. Crystal waters are depicted as red spheres. All residues are in *Neisseria gonorrhoeae* CA numbering.

range, of 3–23 nM for activating SazCA (Table 1). It may be noted that all these compounds have a rather similar scaffold, with the bulky aromatic ring (phenyl, 3,4-dihydroxyphenyl or substituted indolyl) to which the aminoethyl or CH–(NH_2)COOH moiety is attached. It should be noted also that the two enantiomers of the same amino acid, show a rather different activating capacity against SazCA, an effect already observed for the activation of other CAs by amino acids.[15–18] For example, L-DOPA **5** is 222.5 times a better SazCA activator compared to D-DOPA **6**. D-Phe is on the other hand 6.9-times a better SazCA activator compared to its enantiomer L-Phe. In the case of the L-/D-Trp pair, the L-enantiomer is 7.66 times a weaker activator compared to D-Trp (Table 1).

(ii) A large number of the remaining amines and amino acids also showed effective SazCA activating properties. Among them, L-His, D-His, L-Phe, L-Tyr, 4-amino-Tyr, 2-pyridylmethylamine **15**, 1-(2-aminoethyl)-morpholine **17** and L-adrenaline **19**, showed K_As in the range of 52–90 nM, being only slightly less effective CAAs compared to the derivatives discussed above. SAR is thus rather obvious, since both amines and amino acids possessing a variety of scaffolds lead to effective activators of this enzyme.

(iii) The least effective SazCA activators were compounds **6** (D-DOPA), **10** (D-Tyr), **16** and **18** (heterocyclic amines), which showed K_As in the range of 0.11–1.15 μM, being thus anyhow rather effective CAAs (compared to the activation of the mammalian isoforms hCA I and II, for which activation constants in the range of 40–45 μM have been measured with some of these CAAs, see Table 2).

(iv) The SazCA activation profile is very characteristic to this isoform, being somehow similar to that of the other extreme-CA investigated earlier, SspCA,[19] but very different from the activation profiles of the mammalian isoforms hCA I and II (Table 2).

SazCA belongs to the α-CA class, to which the human isoforms hCA I and II also belong. In fact, as shown in our previous work,[14] all amino acid residues involved in the catalytic cycle are conserved in this bacterial isoform and the corresponding mammalian ones, although more than 800 million years of independent evolution separate these organisms. Thus, it is presumable that the activation mechanism reported earlier for the mammalian isoforms is also valid for SazCA. Naturally, His64, an amino acid residue conserved in the bacterial and mammalian isoforms acts as a proton shuttle residue between the active site and the environment, transferring a proton from the zinc-coordinated water molecule through the imidazolic moiety.[18,23] When activators are present, additional proton transfer pathways may take place through the protonatable moiety from the activator molecule (amino, imidazole, carboxylate moieties, etc.).[18,23] To illustrate this, we have docked histamine (N^{τ}–H tautomer; imidazole NE2 protonated) into a SazCA homology model based on the *Neisseria gonorrhoeae* CA crystal structure (NgCA; PDB: 1KOQ; 1.90 Å).[24] The crystal waters in the active site, including the Zn^{2+}-bound water and the 'deep water', were retained. The homology model was constructed using MOE (version 2011.10, CCG, Montreal, Canada), while dockings were performed with the GOLD Suite software package (version 5.1, CCDC, Cambridge, UK) and ChemScore scoring function (using 25 dockings per ligand and default settings).

As shown in Fig. 1, histamine can bind in several ways at the entrance of the active site cavity, not far away from the natural proton shuttle residue (His93 in NgCA; His64 in hCA II), in the so-called activator binding site (as proved by X-ray crystallography on adducts of hCA I and II with many such CAAs).[15–18] The docked poses of histamine form hydrogen bonds with the side chains of Gln116 and Thr204 and the backbone carbonyl of Pro205 (NgCA numbering; corresponding to Gln92, Thr200 and Pro201 in hCA II). Histamine is located at the entrance of the active site cavity and is separated from the zinc-coordinated water molecule by at least 1 water molecule in our docking. For other activators we suggest that a network of 2–4 water molecules may separate the activator from the zinc-coordinated water molecule.[15–18] Their binding is probably also stabilized by interactions with amino acids residues from the active site. However, these interactions were not shown in Figure 1 as they are particular to each activator investigated so far, and to each CA isoform.[15–18,23]

In conclusion, we investigated the α-CA, from the extremophilic bacterium *Sulfurihydrogenibium azorense* for its activation with a series of amino acids and amines. The best SazCA activators were D-Phe, L-DOPA, L- and D-Trp, dopamine and serotonin, which showed activation constants in the range of 3–23 nM. L- and D-His, L-Phe, L-Tyr, 2-pyridyl-methylamine and L-adrenaline were also effective activators (K_As in the range of 62–90 nM), whereas D-Dopa, D-Tyr and several heterocyclic amines showed activity in the micromolar range. A homology modelling of this enzyme in

adduct with histamine, proves that the activator binding site in the bacterial CAs is similar to the one of mammalian enzymes. Indeed, histamine was found bound at the entrance of the active site cavity, interacting with amino acid residues Gln116, Thr204 and Pro205. The good thermal stability, robustness, very high catalytic activity and propensity to be activated by simple amino acids and amines, make SazCA a very interesting candidate for biomimetic CO_2 capture processes. Work is in progress in our laboratories for finding an efficient system for CO_2 capture with SazCA, eventually in the presence of activators of the type investigated here.

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20. Khalifah, R. G. *J. Biol. Chem.* **1971**, *246*, 2561. An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalysed CO_2 hydration activity. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nM, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M $NaClO_4$ (for maintaining constant the ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and activation constants. For each activator at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of activators **1–19** (10 mM) were prepared in distilled-deionized water and dilutions up to 0.001 μM were done thereafter with the assay buffer. Activator and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–A complex. The activation constant (K_A), defined similarly with the inhibition constant K_I, [21] may be obtained by considering the classical Michaelis-equation Eq. (1), which has been fitted by non-linear least squares by using PRISM 3:

$$v = v/\{1 + K_M/[S](1 + [A]_f/K_A)\} \quad (1)$$

where $[A]_f$ is the free concentration of activator.
Working at substrate concentrations considerably lower than K_M ($[S] << K_M$), and considering that $[A]_f$ can be represented in the form of the total concentration of the enzyme ($[E]_t$) and activator ($[A]_t$), the obtained competitive steady-state equation for determining the activation constant is given by Eq. (2): [21]

$$v = v_0 \cdot K_A/\{K_A + ([A]_t - 0.5\ \{([A] + [E]_t + K_A([A]_t + [E]_t + K_A)^2 - 4[A]_t \cdot [E]t)^{1/2}\}\} \quad (2)$$

where v_0 represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator.[21]
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